

A DNA enzyme with Mg²⁺-dependent RNA phosphoesterase activity

Ronald R Breaker[†] & Gerald F Joyce^{*}

Departments of Chemistry and Molecular Biology, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037, USA

Background: Previously we demonstrated that DNA can act as an enzyme in the Pb²⁺-dependent cleavage of an RNA phosphoester. This is a facile reaction, with an uncatalyzed rate for a typical RNA phosphoester of ~10⁻⁴ min⁻¹ in the presence of 1 mM Pb(OAc)₂ at pH 7.0 and 23 °C. The Mg²⁺-dependent reaction is more difficult, with an uncatalyzed rate of ~10⁻⁷ min⁻¹ under comparable conditions. Mg²⁺-dependent cleavage has special relevance to biology because it is compatible with intracellular conditions. Using *in vitro* selection, we sought to develop a family of phosphoester-cleaving DNA enzymes that operate in the presence of various divalent metals, focusing particularly on the Mg²⁺-dependent reaction.

Results: We generated a population of >10¹³ DNAs containing 40 random nucleotides and carried out repeated rounds of selective amplification, enriching for molecules

that cleave a target RNA phosphoester in the presence of 1 mM Mg²⁺, Mn²⁺, Zn²⁺ or Pb²⁺. Examination of individual clones from the Mg²⁺ lineage after the sixth round revealed a catalytic motif comprised of a three-stem junction. This motif was partially randomized and subjected to seven additional rounds of selective amplification, yielding catalysts with a rate of 0.01 min⁻¹. The optimized DNA catalyst was divided into separate substrate and enzyme domains and shown to have a similar level of activity under multiple turnover conditions.

Conclusions: We have generated a Mg²⁺-dependent DNA enzyme that cleaves a target RNA phosphoester with a catalytic rate ~10⁵-fold greater than that of the uncatalyzed reaction. This activity is compatible with intracellular conditions, raising the possibility that DNA enzymes might be made to operate *in vivo*.

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Introduction

The isolation, by *in vitro* selection [1-3], of a family of DNA enzymes that exhibit Pb²⁺-dependent RNA phosphoesterase activity [4], confirmed earlier speculation [5] that DNA could function like protein- and RNA-based enzymes. More recently, *in vitro* selection was used to isolate a family of DNA enzymes that catalyze the joining of two DNA oligomers, one bearing a 5' hydroxyl and the other a 3' imidazole-phosphate [6], suggesting that DNA may be capable of an extensive range of catalytic activities. Complex secondary and tertiary structures can be formed by single-stranded DNA [7-11] and its analogs [12-14]. In addition, chimeric RNA-DNA molecules can form active hammerhead ribozymes [15,16], and an example exists of a Cu²⁺-dependent self-cleaving DNA [17]. Here we report the isolation of new RNA phosphoester-cleaving DNA enzymes that function with an expanded range of divalent metal ion cofactors, and we describe the characteristics of a catalytic DNA that operates under physiological conditions.

A number of different self-cleaving ribozyme motifs [18] have been isolated from natural sources and have been studied for their potential use as sequence-specific 'catalytic antisense' agents [19,20]. Each requires Mg²⁺ or some other divalent metal cation as a cofactor to accelerate the cleavage of a particular RNA phosphoester. We applied *in vitro* selection to large pools of

random-sequence DNA, screening for molecules that catalyze this same reaction in the presence of various divalent metals. We focused mainly on the Mg²⁺-dependent reaction because of its biochemical interest and potential relevance to the cellular environment. The pK_a of a Mg²⁺-bound water is 11.4, making this a challenging reaction at neutral pH. A Mg²⁺-dependent DNA enzyme that operates *in vivo* would be required to function in the presence of 0.5-3.5 mM free Mg²⁺ [21]. Accordingly, DNAs were selected for their ability to cleave a target RNA phosphoester at pH 7.0 in the presence of 1 mM Mg²⁺.

Results and discussion

A population of >10¹³ different double-stranded DNAs, each containing a 5'-biotin and a single embedded ribonucleotide (Fig. 1a), was immobilized on a streptavidin column and subsequently denatured to produce a matrix that displays single-stranded molecules. These molecules also contained a 40-nucleotide random-sequence domain, flanked by two pairing regions that were intended to function as substrate-binding domains. RNA-cleaving DNAs were isolated by eluting the column with buffered solutions containing 1 mM of either Mg²⁺, Mn²⁺, Zn²⁺ or Pb²⁺, in sequential order. The selected DNAs were amplified separately by the polymerase chain reaction (PCR) and the 5'-biotin and embedded ribonucleotide were reintroduced by additional PCR amplification

*Corresponding author. †Present address: Department of Biology, Yale University, New Haven, CT 06520-8103, USA.

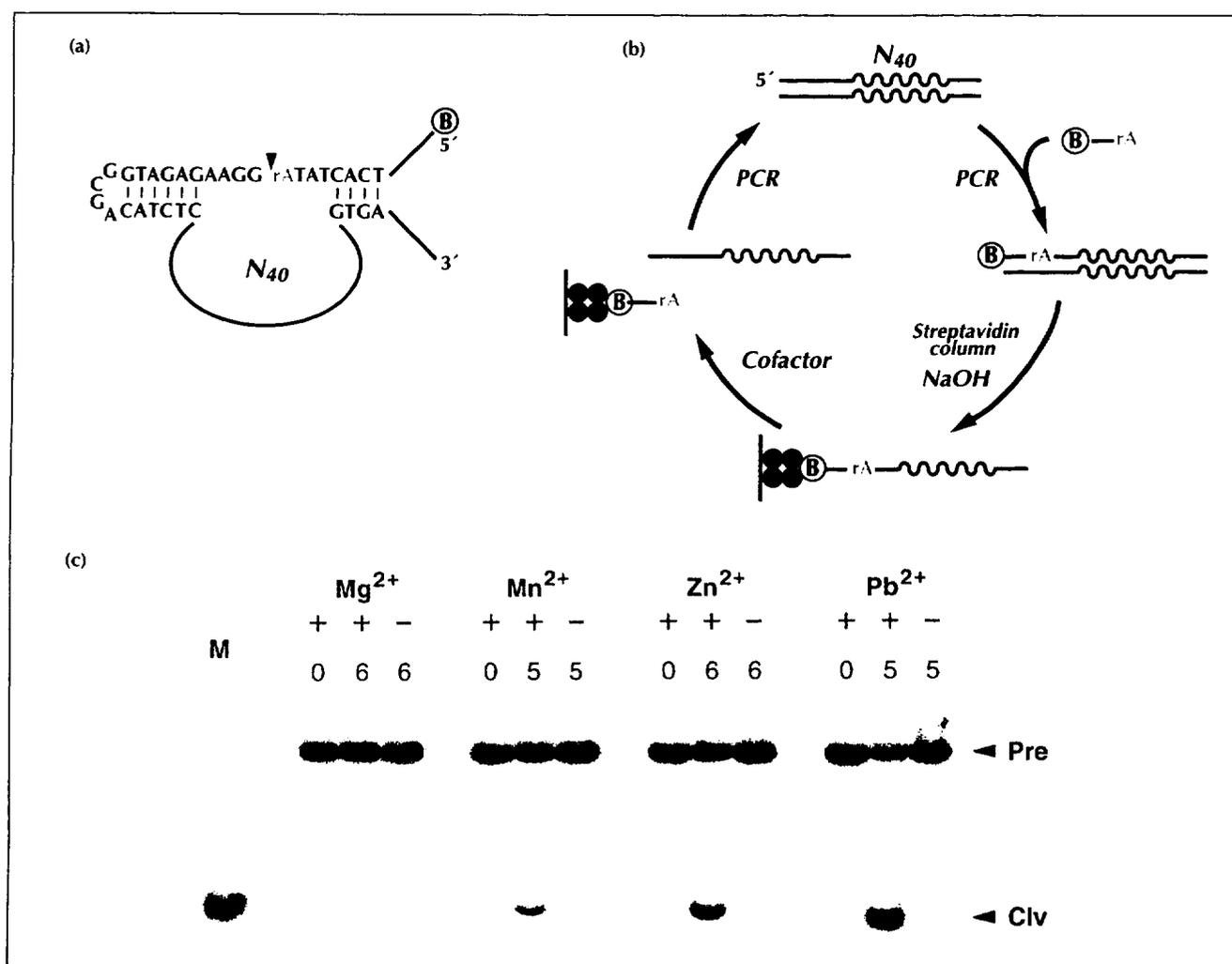


Fig. 1. Isolation of DNAs that catalyze the metal-dependent cleavage of an RNA phosphoester. (a) Starting pool of random-sequence DNAs, engineered to contain two substrate-binding domains. Each member of the pool contains a 5'-terminal biotin (encircled B), a single embedded ribonucleotide (rA) and a 40-nucleotide random-sequence domain (N₄₀). (b) Selective-amplification scheme for isolation of DNAs that catalyze the cofactor-dependent cleavage of an RNA phosphoester. (c) Self-cleavage activity of the starting pool of DNA (0) and populations obtained after either the fifth (5) or sixth (6) rounds of selective amplification, for each of the four metal-dependent lineages. [5'-³²P]-labeled precursor (Pre) was incubated at 23 °C for 1 h in buffer B with either none (-) or 1 mM (+) added divalent metal ion, as indicated. M is [5'-³²P]-labeled primer 3, corresponding in sequence to the expected 5' cleavage product (Clv).

(Fig. 1b). The entire selective-amplification procedure was carried out repeatedly for each of the four lineages.

After five (Mn²⁺ and Pb²⁺) or six (Mg²⁺ and Zn²⁺) rounds, all four lineages showed catalytic activity in the presence of their respective metal cofactor (Fig. 1c). Product formation under the selection conditions for the Mg²⁺, Mn²⁺, Zn²⁺ and Pb²⁺ populations was 3 %, 11 %, 24 % and 62 %, respectively (Table 1). These values are inversely proportional to the pK_a values of the corresponding metal-bound water (11.4, 10.6, 9.0 and 7.7, respectively). A similar relationship has been seen when substituting other divalent metals for Mg²⁺ with the hammerhead ribozyme [22], in both cases suggesting that the metal hydroxide is directly involved in catalysis.

Examination of 25 clones [23] from each lineage revealed that the engineered substrate pairing incorporated

into the original pool was frequently disrupted by mutations that accumulated during the selective-amplification process. Many individuals from each of the selected populations have sequence similarity to the Pb²⁺-dependent

Table 1. Activities of four catalytic DNA lineages.

Lineage	Metal ^a			
	Mg ²⁺	Mn ²⁺	Zn ²⁺	Pb ²⁺
Mg ²⁺	3	32	50	51
Mn ²⁺	0	11	21	49
Zn ²⁺	0	11	24	42
Pb ²⁺	0	2	5	62

^aShows % of population cleaved when 10 nM precursor was incubated for 1 h with 1 mM metal ion. Conditions as for Fig. 1c.

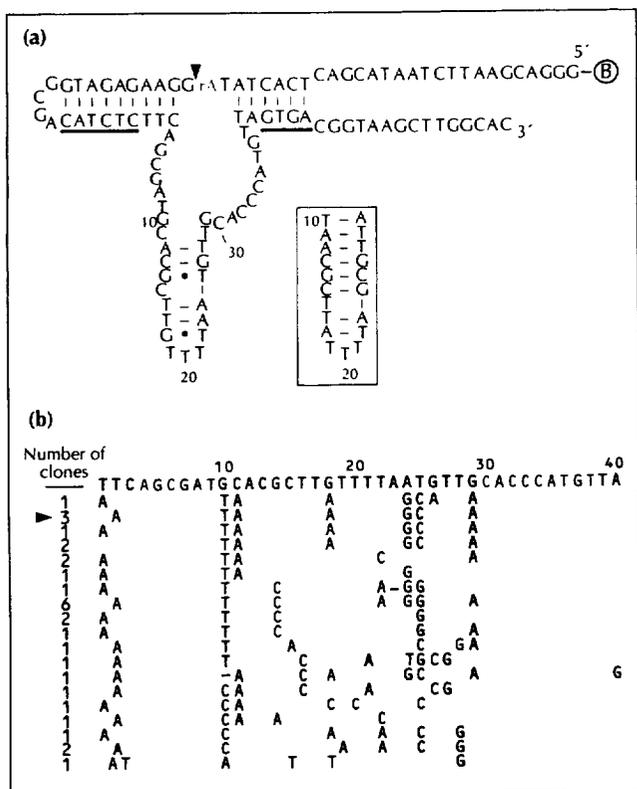


Fig. 2. Mg^{2+} -dependent DNA catalysts. (a) Sequence and predicted secondary structure of the most active catalyst isolated from the Mg^{2+} -dependent lineage after six rounds of selective amplification. Underlined sequences correspond to the engineered substrate-binding domains included in the original pool design (Fig. 1a). Conserved residues are shown in green. Boxed hairpin depicts the optimized structure of a representative clone obtained after reselection. (b) Sequence alignment of individuals isolated after randomization of the most active Mg^{2+} -dependent clone and seven additional rounds of selective amplification. The sequence of each clone matches the prototype sequence except where noted. The arrowhead identifies the clone shown in the inset in (a).

DNA enzyme described previously [4]. The clones isolated from the Mg^{2+} lineage, however, have a more complex structure composed of a three-stem junction. All of these clones have very similar sequences, with only four being unique. The most active clone, which occurred five times, is shown in Figure 2a. Under the selection conditions, this individual has a first-order reaction rate, k_{obs} , of 0.002 min^{-1} . It is 3-, 19- and 5-fold more active in the presence of 1 mM Mn^{2+} , Zn^{2+} and Pb^{2+} , respectively.

We prepared a degenerate pool of synthetic DNA based on the sequence of this most-active clone, randomizing the 40 nucleotides that lie between the engineered pairing domains such that the original nucleotide occurred with a probability of 0.85 and each of the other three nucleotides occurred with a probability of 0.05. We carried out seven additional rounds of selective amplification, based on activity in the presence of 1 mM Mg^{2+} , and examined 30 clones from the resulting population (Fig. 2b). This revealed one highly variable and two strictly conserved regions within the

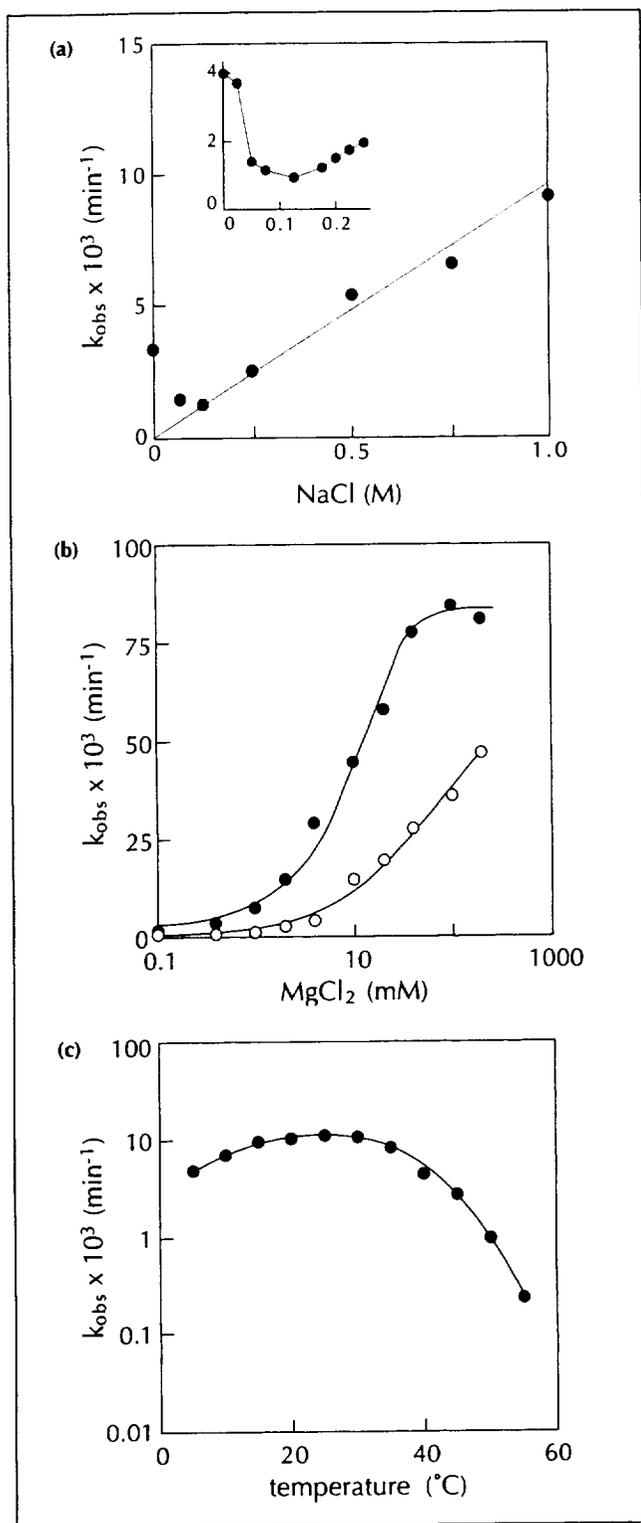


Fig. 3. Characterization of the intramolecular reaction catalyzed by the optimized catalyst. All reaction mixtures contained $10 \text{ nM } [5\text{-}^{32}\text{P}]\text{-labeled precursor}$ and were incubated at 23°C in $50 \text{ mM HEPES (pH 7.0)}$, unless otherwise indicated. (a) Effect of NaCl concentration on k_{obs} in the presence of 1 mM MgCl_2 . The inset displays a detail of the data for $0\text{--}0.25 \text{ M NaCl}$. (b) Effect of MgCl_2 concentration on k_{obs} in the presence of 1 M (●) or no (○) NaCl . (c) Temperature dependence of k_{obs} in the presence of 10 mM MgCl_2 and 1 M NaCl . Precursor DNA was prepared by either 5 cycles (for (a) or (b)) or 20 cycles (for (c)) of PCR amplification (see Materials and methods).

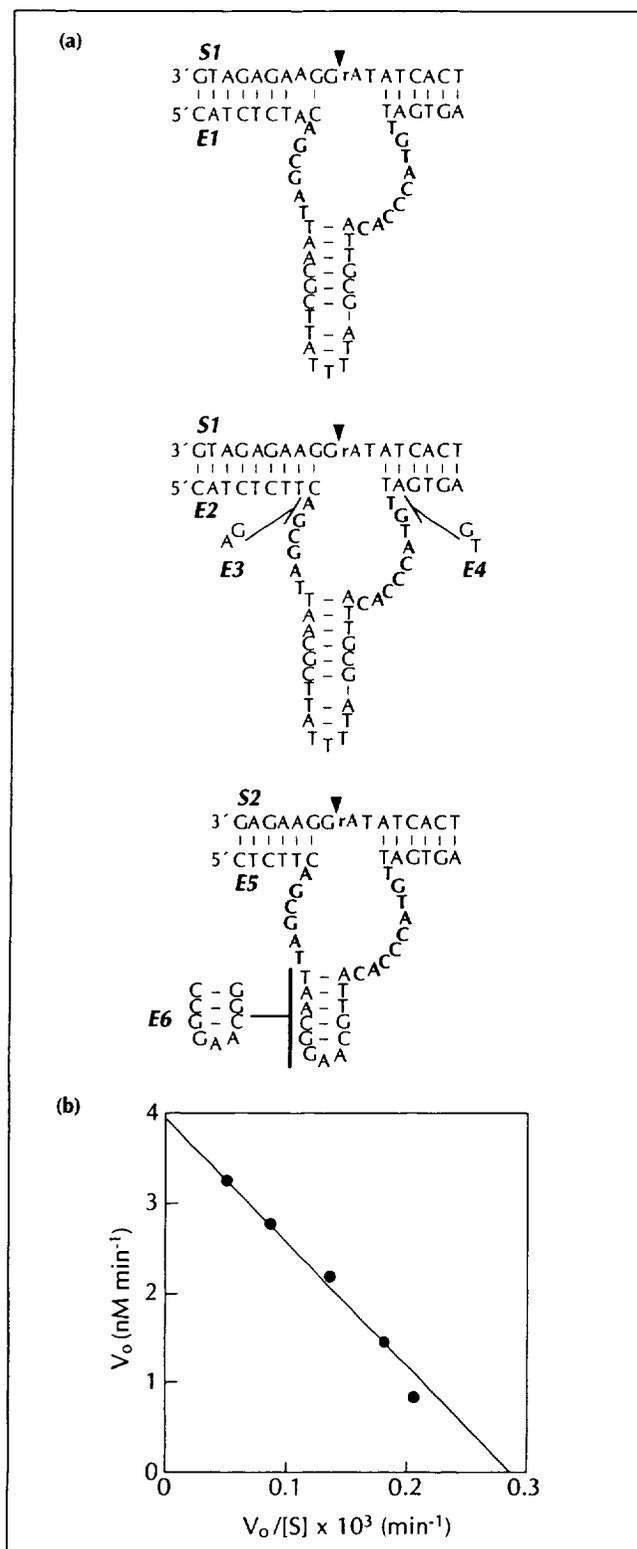


Fig. 4. (a) Sequence and secondary structure of complexes formed between various substrates and enzymes. Conserved residues are shown in green. (b) Eadie-Hofstee plot for the reaction involving S2 and E6. Initial rates of cleavage were determined for reaction mixtures containing 100 nM E6 and either 4, 8, 16, 32 or 64 μ M S2.

40-nucleotide randomized domain. The sequence variation at positions 10–29 is consistent with the existence of a stem-loop structure.

One of the optimized DNA catalysts isolated from the reselected pool (Fig. 2a, inset) has a k_{obs} of 0.01 min^{-1} in the presence of 1 mM Mg^{2+} and 0.02 min^{-1} in the presence of 1 mM Pb^{2+} . Addition of $1\text{--}10 \mu\text{M Pb}^{2+}$ to a reaction mixture containing 1 mM Mg^{2+} resulted in no significant increase in the catalyzed rate of phosphoester cleavage. The rate of the Mg^{2+} -dependent reaction is 10^5 -fold enhanced compared to the uncatalyzed rate of cleavage of 5'-GTAGAGAAGG rA TATCACT, measured under the same reaction conditions. This rate enhancement approaches that observed for the hammerhead ribozyme [24].

At low concentrations, NaCl inhibits the rate of catalysis, but the rate increases linearly with increasing concentrations of NaCl above 0.1 M (Fig. 3a). This inhibitory effect is not detected when 10 mM Mg^{2+} is used. A plot of activity versus Mg^{2+} concentration reveals a K_M for Mg^{2+} of 10 mM , with a maximum catalytic rate of 0.08 min^{-1} reached at saturation (Fig. 3b). The rate of RNA phosphoester cleavage is largely independent of temperature over the range of $15\text{--}35^\circ\text{C}$, but drops sharply at higher temperatures (Fig. 3c). The DNA catalyst has a broad pH optimum, with $k_{\text{obs}} \sim 10^{-2} \text{ min}^{-1}$ between pH 6.2 and 8.6 in the presence of 10 mM MgCl_2 and 1 M NaCl . Under simulated physiological conditions (2 mM MgCl_2 , 150 mM KCl , 50 mM HEPES (pH 7.4), 37°C) the rate of RNA phosphoester cleavage is 0.001 min^{-1} .

The optimized DNA catalyst was divided into separate substrate and enzyme domains, which were tested for activity in an intermolecular context (Fig. 4a). The oligomers S1 and E1 correspond precisely to a truncated version of the optimized DNA catalyst; E2 contains a single A to T change that repairs a putative A•A mismatch within one of the two substrate-binding domains. Both E1 and E2 catalyze the cleavage of S1, yielding a 5' product with a phosphate at its 3' terminus and a 3' product with a free 5' hydroxyl. Under multiple turnover conditions, employing $20 \mu\text{M S1}$ and $0.2 \mu\text{M E2}$, $3.4 \mu\text{M}$ products were formed after a 5 h incubation in the presence of 10 mM MgCl_2 and 1 M NaCl at pH 7.0 and 23°C , corresponding to 17 turnovers of the enzyme. This rate closely approximates k_{obs} for the unimolecular self-cleavage reaction.

Constructs E3 and E4 (Fig. 4a) showed no catalytic activity, suggesting that mutations within either of the two strictly-conserved unpaired regions are not tolerated. Truncation of the substrate (S2), and either shortening or altering the sequence of the central hairpin of the enzyme (E5, E6), does not significantly alter enzymatic activity. Cleavage of S2 by E6, in the presence of 10 mM MgCl_2 and 1 M NaCl , proceeds with a k_{cat} of 0.039 min^{-1} and K_M of $13 \mu\text{M}$ (Fig. 4b). E6 is unable to cleave either an all-DNA or all-RNA analog of S2.

The Mg^{2+} -dependent DNA enzyme developed here is structurally more complex than the Pb^{2+} -dependent

molecule generated previously [4]. The latter compound is comprised of two stem structures that bind the substrate, surrounding a central core of 15 nucleotides. Six of the core nucleotides are highly conserved. The Mg^{2+} -dependent compound also contains two stems that are involved in substrate binding and has a central core containing 15 highly-conserved nucleotides and a stem-loop structure of variable sequence. The secondary structure of the Mg^{2+} -dependent DNA enzyme is similar to that of the hammerhead ribozyme, which also catalyzes the Mg^{2+} -dependent cleavage of an RNA phosphoester. There is no reason to believe, however, that the two molecules adopt similar tertiary structures.

The Mg^{2+} -dependent DNA enzyme provides a rate enhancement of $\sim 10^5$ compared to the uncatalyzed reaction. This is similar to the rate enhancement observed for the Pb^{2+} -dependent DNA enzyme [4] and is about 10-fold less than that of the hammerhead ribozyme operating in the presence of Mg^{2+} [25]. The catalytic activity of both the Mg^{2+} -dependent DNA enzyme and hammerhead ribozyme is significantly lower at physiological $MgCl_2$ concentrations (0.5–3.5 mM) than at a $MgCl_2$ concentration of 10 mM (Fig. 3b) [22]. We have made no attempt to improve the Mg^{2+} -binding affinity of the DNA enzyme, for example, by carrying out *in vitro* selection at very low $MgCl_2$ concentrations. Such optimization is best deferred until DNA enzymes are developed that cleave a target site within a segment of RNA that is of biological interest.

Significance

We have shown that DNA enzymes can use a variety of metal ion cofactors and can catalyze a biologically-relevant reaction under conditions similar to those that exist within cells. It is now clear that the occurrence of DNA enzymes in living systems, either extant or extinct, cannot be ruled out based on any perceived physical and chemical inadequacies of DNA itself. Moreover, it may now be practical to tailor-make, through *in vitro* selection, RNA-cleaving DNA enzymes that specifically target cellular RNAs and that can be used in a therapeutic capacity as an alternative to ribozymes.

Materials and methods

Oligonucleotides and oligonucleotide analogs

Synthetic DNAs and DNA analogs were purchased from Operon Technologies. Primer 3a, [5'- ^{32}P]-GGGACGAATTCTAATACGACTCACTATrA-3', was prepared by 5'-labeling primer 3 using [γ - ^{32}P]ATP and T4 polynucleotide kinase. S1 was prepared as described previously [4] and S2 was purchased from Oligos Etc.

DNA pool preparation and in vitro selection

The starting pool of DNA was generated by extending 150 pmoles of primer 3b, 5'-biotin-GGGACGAATTCTAATA-

CGACTCACTATrA-3', in the presence of an equal amount of template, 5'-GTGCCAAGCTTACCGTCAC-N₄₀-GAGATGTCGCCATCTCTTCCTATAGGAGTCGT-ATTAG-3' (N indicates equal representation of G, A, T and C), in a 300- μ l reaction mixture containing 0.2 U μ l⁻¹ *Taq* DNA polymerase, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3 at 23 °C), 0.01% gelatin and 0.2 mM of each dNTP, for 1 min at 92 °C, 1 min at 50 °C and 10 min at 72 °C. The extended DNA was recovered [4], resuspended in buffer A (1 M NaCl, 50 mM HEPES (pH 7.0), 0.02 mM EDTA) and immobilized by passing the solution through three successive streptavidin affinity columns. The columns were washed with five 100- μ l volumes of buffer A and five 100- μ l volumes of 0.2 N NaOH, equilibrated with buffer A, eluted over the course of 1 h with three 20- μ l aliquots of buffer B (1 M NaCl, 50 mM HEPES (pH 7.0)) with added 1 mM $MgCl_2$ and again washed with five 100- μ l volumes of buffer A. The process was repeated, successively replacing the $MgCl_2$ with 1 mM $MnCl_2$, $ZnCl_2$ and $Pb(OAc)_2$, thereby creating four separate DNA lineages. DNA was recovered from each eluate by precipitation with ethanol following addition of 20 pmoles each of primer 1, 5'-GTGCCAAGCTTACCG-3', and primer 2, 5'-CTGCAGAATTCTAATACGACTCACTATAGGAAGAGATGGCGAC-3'. Each of the four recovered populations of DNA was amplified separately by PCR in a 100- μ l volume over 25 temperature cycles [4]. 10 μ l of the resulting mixture was used to initiate a 100- μ l nested PCR containing 50 pmoles each of primer 1 and primer 3b, which was amplified over six temperature cycles. Selective amplification was carried out for five successive rounds with Mn^{2+} and Pb^{2+} , with reaction times of 1 h (rounds 1–3), 20 min (round 4) and 1 min (round 5), and for six rounds with Mg^{2+} and Zn^{2+} , with reaction times of 1 h (rounds 1–4), 20 min (round 5) and 1 min (round 6). Rounds 1–3 of the reselection involved reaction in buffer B with added 1 mM $MgCl_2$ for 1 h. Rounds 4–7 involved pre-reacting with buffer B with added 1 mM $Pb(OAc)_2$ for 1 h, followed by reaction with buffer B with added 1 mM $MgCl_2$ for 1 h (round 4), 20 min (rounds 5 and 6) or 1 min (round 7).

Preparation of precursor DNAs

Precursor molecules, from either a population of DNAs or an individual clone, were prepared with a 5'- ^{32}P label by PCR amplification in a 25- μ l reaction mixture containing 10 pmoles each of primer 1b (5'-biotinylated form of primer 1) and primer 3a, 0.5 pmoles input DNA and 0.1 U μ l⁻¹ *Taq* polymerase, which was incubated for either 5 or 20 cycles of 1 min at 92 °C, 1 min at 50 °C and 1 min at 72 °C under the conditions described above. Precursor DNA prepared by 20 cycles of PCR amplification consistently showed 4–5-fold lower activity than that prepared by 5 cycles of amplification. Following PCR, NaCl was added to a final concentration of 1 M and the entire mixture was passed through a streptavidin column. The column was washed with buffer A and the single-stranded precursor DNA was recovered by elution with 40 μ l of 0.1 N NaOH, neutralized with an excess of sodium acetate (pH 5.2), precipitated with ethanol and purified by denaturing polyacrylamide gel electrophoresis.

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